Attachment #9

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6: G01N 33/68, 33/50 — A1

(11) International Publication Number: WO 97/07402

(43) International Publication Date: 27 February 1997 (27.02.97)

(21) International Application Number: PCT/CA96/00555

(22) International Filing Date: 16 August 1996 (16.08.96)

(30) Priority Data:
08/515,615 17 August 1995 (17.08.95) US

(71) Applicant: THE ONTARIO CANCER INSTITUTE [CA/CA]; 610 University Avenue, Toronto, Ontario M5G 2M9 (CA).

(72) Inventor: CHAKRABARTTY, Avi; Apartment #710, 400 Walmer Road, Toronto, Ontario M5P 2X7 (CA).

(74) Agents: LAKE, James, R. et al.; Ridout & Maybee, Suite 2400, One Queen Street East, Toronto, Ontario M5C 3B1 (CA).

(81) Designated States: AU, CA, CN, JP, KR, MX, NZ, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PROTEIN FIBRIL ASSEMBLY ASSAY

(57) Abstract

The invention is a method for *in vitro* monitoring of peptide or protein fibril assembly. In order to provide sensitivity at nanomolar concentrations on the order of that observed *in vivo*, the method makes use of fluorescent energy transfer between closely juxtaposed donor and acceptor fluorophores. Accordingly, the invention requires attaching a donor fluorophore to a fibrillogenic peptide or protein, and attaching an acceptor fluorophore to a second fibrillogenic peptide or protein. The donor and acceptor fluorophores are located on the first and second peptides or proteins so that they are juxtaposed to permit energy transfer between them upon fibril formation. The fluorophore-containing peptides or proteins are mixed in solution at a normal physiological concentration, and fibril formation is monitored by observing the fluorescence energy transfer between the donor and acceptor fluorophores. Mixing is preferably done by providing equimolar amounts of peptides or proteins in a denaturant solution, and monitoring of fibril formation is initiated by diluting out the denaturant.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia
AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

PROTEIN FIBRIL ASSEMBLY ASSAY

The invention is a method for *in vitro* monitoring of protein fibril assembly at physiologically relevant concentrations. The invention is readily adaptable for *in vitro* monitoring of fibril assembly processes associated with various amyloidosis disorders, such as Alzheimer's disease, multiple myeloma, rheumatoid arthritis, diabetes, and prion disorders. The invention has particular utility as a screening assay for potential inhibitors of fibril formation, which in turn may be candidates for treatment of Alzheimer's disease or other amyloidosis disorders.

Alzheimer's disease is a neurodegenerative disorder of the elderly. While neurodegeneration in Alzheimer's disease begins slowly, its progression follows an exponential course, and it finally culminates in dementia and death (Muller-Hill and Beyreuther, 1989). Key pathological features of Alzheimer's disease found in the brains of afflicted patients include the following: a) senile plaques, which are extracellular amyloid deposits in close contact with neurons, b) neurofibrillary tangles, which are intraneuronal deposits of paired helical filaments, and c) cerebrovascular amyloid, which are amyloid deposits in the walls of cortical and meningeal vessels (Selkoe, 1991; Katzman and Saitoh, 1991). The link between the symptoms of Alzheimer's disease and its pathology arises from the plaques and neurofibrillary tangles being concentrated around the hippocampus, a region of the brain responsible for memory and learning.

A major component of senile plaques and cerebrovascular amyloid is Alzheimer's β-amyloid peptide (Aβ), a 39-43 residue peptide that folds into a β-sheet structure and assembles into fibrils 60-90Å in diameter

(Fraser et al., 1993). Aβ is a fragment of a much larger integral membrane protein called amyloid precursor protein (APP). APP possesses one transmembrane segment, a large extracellular domain, a small intracellular domain, several glycosylation and sulfation sites, and a Kunitz protease inhibitor domain. Aβ is derived from residues 672 to 715 of APP, and it encompasses the C-terminal region of the extracellular domain of APP and the N-terminal half of the transmembrane domain of APP. Other proteins found in senile plaques include: serum complement proteins (C1q, C3d, and C4d), serum amyloid P, α_1-antichymotrypsin, Apo-E, and heparan sulfate proteoglycans. The relative amounts of these proteins in senile plaques are, however, much lower than that of Aβ.

The other characteristic feature of Alzheimer's disease are neurofibrillary tangles, which are intraneuronal deposits of paired helical filaments. Paired helical filaments are not as well characterized as senile plaques (Crowther, 1991). The protein components of paired helical filaments discovered so far include: neurofilament protein, a microtubule associated protein (MAP) called tau, MAP2, and MAP5. Recent evidence suggests that paired helical filament formation results, at least in part, from abnormal phosphorylation of tau.

The molecular pathogenesis of Alzheimer's disease is not well established. However, there is accumulating evidence that deposition of Aβ-fibrils in senile plaques may account for much of the neurotoxicity of Alzheimer's disease (Selkoe, 1993). Neurons which contact Aβ-fibrils are dystrophic. Patients that have three copies of the APP gene (i.e. Down's syndrome patients) produce a greater number of senile plaques and develop Alzheimer's disease when they reach their late thirties. Mutations in the APP gene are often seen in cases of familial Alzheimer's disease, and related genetic diseases (Goate

et al., 1991). Aβ-fibrils are toxic to neurons in culture (Yankner et al., 1989; Pike et al., 1993). Alzheimer-type neuropathology is induced in transgenic mice overexpressing a mutant form of APP (Games et al., 1995). These findings all point to Aβ as the primary neurotoxic agent of Alzheimer's disease. The mechanism of Aβ neurotoxicity is also not well understood, but it appears to be related to calcium ion homeostasis in neurons (Mattson et al., 1993).

Two experimental findings that must be reconciled by any proposed model of Alzheimer's disease pathogenesis is that Aβ is a product of normal cells (Haass et al., 1992), and that it is present in cerebrospinal fluid and blood plasma of healthy individuals (Seubert et al., 1992). Experimental evidence suggests that there may be multiple primary causes that ultimately lead to Aβ deposition in senile plaques and Alzheimer's disease (Selkoe, 1993). While certain individuals may develop Alzheimer's disease by producing greater amounts of Aβ, others might develop the disease by producing a form of Aβ that is especially prone to fibril formation. Thus, the concentration of Aβ in the brain as well as its intrinsic tendency to aggregate are both integral to the process of Aβ-fibril deposition in Alzheimer's disease.

Given the possibility that fibril formation by Aβ may account for the neurotoxicity in Alzheimer's disease, it follows that molecules that inhibit fibril formation should be good candidates for therapeutic agents for the treatment of Alzheimer's disease. One critical research tool for evaluating therapeutic inhibitors is an assay for Aβ-fibril formation. Current methods that monitor Aβ fibril formation employ various biophysical techniques, such as electron microscopy, X-ray diffraction, fourier transform infrared (FTIR) spectroscopy, circular dichroism (CD) spectroscopy, and nuclear magnetic

resonance (NMR) spectroscopy. Studies utilizing these biophysical techniques have shown that Aβ fibrils possess remarkable intrinsic stability. Even after altering much of the sequence of Aβ, fibril formation still occurs (Fraser et al., 1992; Hilbich et al., 1992; Fraser et al., 1994). Asp23 of Aβ, however, is a unique residue; since when it is replaced with Lys a significant reduction in the stability of the fibril is observed (Fraser et al., 1994). Two regions of the Aβ sequence show a high propensity to form β-structure: residues 9 to 28 (Fraser et al., 1991) and residues 34 to 42 (Halverson et al., 1990). One factor which has a major effect on fibrillogenesis is pH; at low (< 3) and high (>10) pH, fibrillogenesis by Aβ analogues is both slower and less stable (Fraser et al., 1991; 1992). It appears that hydrogen bond, hydrophobic, and electrostatic interactions all act in concert to stabilize the Aβ fibril; however, a high resolution structural model of the Aβ fibril has yet to be developed.

The studies on the mechanism of fibrillogenesis mentioned have employed methods of measuring fibril formation which require sample concentrations that are 200,000 to 5,000,000-fold higher than the physiological concentration. Fibril assembly is a bimolecular reaction: $A\beta + (A\beta)_n = (A\beta)_{n+1}$. One distinguishing feature of bimolecular reactions is that both their kinetics and thermodynamics are concentration-dependent. It follows, therefore, that the concentration of Aβ in the brain and cerebrovasculature controls the rate of amyloid fibril formation as well as the intrinsic stability of amyloid plaques. Consequently, the mechanism of fibril assembly at low physiological concentrations may differ from the mechanism that operates at the high concentrations required by the above mentioned biophysical techniques. Any attempt to model Alzheimer's disease *in vitro* should take into

consideration the relatively low concentrations of Aβ in the brain and cerebrovasculature.

Another limitation of using biophysical techniques, such as electron microscopy, FTIR, CD, and X-ray diffraction, to follow fibril formation is the technical complexity of such techniques. Specialized training and involved procedures are required; thus, limiting the usefulness of these techniques as screening tests for fibrillogenesis inhibitors.

In contrast to prior techniques, the invention provides a very sensitive fluorescence technique for monitoring fibrillogenesis by Aβ at concentrations close to the physiological concentration of Aβ in the brain. This fluorescence technique can be adapted into a rapid, technically simple, standardized assay to simultaneously screen many compounds that could potentially inhibit fibril formation.

The skilled person will appreciate that the method as applied to Aβ fibrillogenesis has general application for the assaying of fibrillogenesis associated with other amyloidosis disorders as mentioned above.

Accordingly, the invention provides a method for *in vitro* monitoring of peptide or protein fibril assembly, comprising the steps of:

1) attaching a donor fluorophore to a first fibrillogenic peptide or protein;

2) attaching an acceptor fluorophore to a second fibrillogenic peptide or protein, the donor and acceptor fluorophores being located on said first and second peptides or proteins so that they are juxtaposed to permit energy transfer between them upon fibril

formation;

3) mixing the first and second fluorophore-containing peptides or proteins in solution at an approximately normal physiological concentration; and

4) monitoring formation of fibrils by observing the occurrence of fluorescence energy transfer between the donor and acceptor fluorophores upon excitation of the donor fluorophores.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows fluorescence emission spectra of fibrils composed of Trp-Aβ(9-25) and AEDANS-Aβ(9-25).

Figure 2 is a graph showing pH dependence of fibril formation monitored by fluorescent fibrillogenesis assay, and attenuation of the titration curve in the presence of 0.5 M NaCl.

Figure 3 is a graph of the kinetics of monomer fibril exchange.

Figure 4 is a graph showing the promotion of fibril formation by heparin as monitored by the fluorescence assay of the invention.

The invention will be described in relation to a specific assay for Aβ fibril assembly. The following description while specific for Aβ fibrillogenesis illustrates the general principles of the invention which may be used to assay fibril assembly processes in other amyloidosis disorders.

Development of a fluorescent fibrillogenesis assay.

Fluorescence spectroscopy is a very sensitive

technique, and depending on the quantum yield of the fluorophore, the technique may be sensitive down to the nanomolar concentration range. The physiological concentration of Aβ in the brain is estimated to be in the nanomolar range; therefore, the sensitivity of fluorescence spectroscopy is in principle sufficient to detect physiological concentrations of Aβ. Unfortunately, fluorescence cannot be used to examine fibril formation by native Aβ because the only intrinsic fluorophore present in Aβ is the phenolic side chain of Tyr10, and its very low quantum yield is inadequate for sensitive measurements. Extrinsic fluorescent groups, however, can be chemically attached to Aβ, and the technique of nonradiative fluoresence energy transfer can be used to measure fibril assembly by Aβ in the nanomolar concentration range.

The principle of fluorescence energy transfer is that when appropriate donor and acceptor fluorophores are close in space, light energy absorbed by the donor can be transferred to the acceptor, and the efficiency of energy transfer depends on the distance separating the donor and acceptor (see Fairclough and Cantor, 1978). The following example outlines the general method of energy transfer measurements. The fluorescent amino acid tryptophan, absorbs light of 281 nm, and after absorption it emits light of 337 nm. The fluorescent compound 5-acetylethyl-diaminonaphthalene-1-sulfonic acid (AEDANS), on the other hand, absorbs light of 281 nm very poorly but it absorbs light of 337 nm very strongly; after absorption AEDANS emits light of 490 nm. Since the absorption band of AEDANS coincides with the emission band of Trp, Trp and AEDANS can be used as donor and acceptor, respectively, in a fluorescence energy transfer experiment. To illustrate the method consider a solution containing a mixture of Trp and AEDANS, if the mixture is irradiated with light of 281 nm then the wavelength of the emitted

light depends on the distance separating the Trp and AEDANS fluorophores. If the two fluorophores are close in space (< 30 Å) then the emitted light will be of 490 nm predominantly; on the other hand, if the chromophores are well separated in space the emitted light will be of 337 nm predominantly. Using Trp and AEDANS as the donor-acceptor pair, a method for monitoring fibril formation by Aβ using nonradiative fluorescence energy transfer was devised.

The fluorescent fibrillogenesis assay of the invention uses a fragment of Aβ corresponding to residues 9 to 25. This fragment of Aβ forms fibrils that display similar morphology, pH-dependence, X-ray diffraction pattern, and secondary structure as the full length Aβ molecule (Fraser et al., 1994). Adapting the assay procedure to the full length Aβ molecule is very straightforward and simple.

Two forms of the fibrillogenic fragment Aβ(9-25), SEQ ID NO:1, were synthesized: one form had a Trp residue appended to the N-terminus of Aβ(9-25) (denoted Trp-Aβ(9-25) SEQ ID NO:2), and a second form (denoted AEDANS-Aβ(9-25) SEQ ID NO:3) had a cysteine residue appended to the N-terminus of Aβ(9-25), and the AEDANS group chemically linked to the sulfhydryl side chain of cysteine (Table 1). The basis of the method is that when Trp-Aβ(9-25) is mixed with AEDANS-Aβ(9-25), fibrils composed of both forms of Aβ will assemble, and in the fibril state the Trp and AEDANS groups will be closer in space than in the nonfibril state. Since fluorescence energy transfer between Trp and AEDANS increases when the two fluorophores are close in space, the efficiency of energy transfer between Trp and AEDANS will increase as Trp-Aβ (9-25) and AEDANS-Aβ (9-25) assemble into fibrils. At a separation distance of 22 Å energy transfer between Trp and AEDANS is easily measurable (Fairclough and

Cantor, 1978; the upper limit of energy transfer is ~40Å).

TABLE 1

Peptide	Amino acid sequence
Aβ(9-25)	GYEVHHQKLVFFAEDVG (SEQ ID NO:1)
Trp-Aβ(9-25)	Ac-WGGYEVHHQKLVFFAEDVG-$CONH_2$ (SEQ ID NO:2)
AEDANS-Aβ(9-25)	Ac-C(AEDANS)GGYEVHHQKLVFFAEDVG-$CONH_2$ (SEQ ID NO:3)

Trp and AEDANS are relatively large chemical structures. There is a possibility, therefore, that introduction of these large groups into Aβ may disrupt or alter fibril formation through steric mechanisms. For this reason, it was decided to place the fluorescent groups at the N-terminus of the Aβ (9-25) sequence rather than at an internal position, because placement in the interior could potentially disrupt certain interactions which are known to stabilize fibril formation (Halverson et al., 1990; Hilbich et al., 1992; Fraser et al., 1994). In addition, a glycine residue was inserted between the fluorescent group and the rest of the Aβ (9-25) sequence. Glycine is the smallest amino acid, it lacks a side chain, and it should act like a flexible tether for the fluorescent group; therefore, as the fluorescent-labeled Aβ (9-25) molecule assembles into fibrils the flexible glycine tether will act like a hinge and allow the fluorescent group to be pushed out of the way if steric clashes occur with other parts of the fibril.

For the fluorescence method of the invention to operate as intended, the fluorescent-Aβ molecules must be prevented from forming fibrils before they are mixed

together. One approach to achieving this objective is to mix the peptides in solid lyophilized form before dissolving them in buffer. The shortcoming of this method, however, is that the relative amounts of each peptide will have to be determined using dry weights, which is a very inaccurate method. An alternative approach is to first dissolve the two peptides in a solution containing a denaturant that prevents fibril formation, and then to initiate the fibril assembly process by diluting out the denaturant. This method will involve: a) preparing concentrated stock solutions of Trp-Aβ and AEDANS-Aβ in denaturant and accurately determining the peptide concentrations by UV absorbance using the absorption coefficients of Trp and AEDANS, b) mixing appropriate amounts of each peptide solution to produce equimolar mixtures of both peptides in denaturant solution, c) initiate fibril assembly by diluting out the denaturant, and d) simultaneously starting to monitor the evolution of fluorescence energy transfer in the fluorimeter. This method should be very rapid and it should allow evaluation of the kinetics and thermodynamics of fibril assembly. The kinetic measurement will be limited only by the dead-time required for diluting out the denaturant, which for manual mixing is around 10 seconds. If the kinetics of fibril assembly occur too rapidly using the manual mixing method, then this method can easily be adapted into a stopped-flow fluorimetric method which has a dead-time of 30 milliseconds.

As regards the choice of denaturant, one possibility is to use formic acid which is known to prevent fibril assembly (Halverson et al., 1990). Initiating fibril assembly, however, would then require neutralization of the acid, which in turn would cause an increase in ionic strength of the solution. Thus fibril assembly at low ionic strengths cannot be explored using an acid as the denaturant. In addition, by placing the peptides in acid

solutions the risks of acid hydrolysis, deamidations, and formylations exist. Therefore, formic acid is not an ideal denaturant for the purposes of the invention. The organic solvent 40% trifluoroethanol has been shown to maintain Aβ in a soluble monomeric helical state at very high peptide concentrations and at pH values between 1.3 and 10 (Barrow and Zagorski, 1991). Accordingly, this solvent was chosen as the preferred denaturant because it does not cause any harmful side reactions, it is nonviscous, so mixing is efficient; and it is volatile allowing for easy removal.

The basic protocol of the fluorescence method of the invention using the described Aβ analogs is: 1) Trp-Aβ(9-25) and AEDANS-Aβ(9-25) are dissolved separately in 40% trifluoroethanol at millimolar concentrations. Under these conditions the Aβ analogues will adopt monomeric helical conformations. 2) After determining the concentration of the stock peptide solutions by UV absorbance, an equimolar mixture of the peptides at micromolar concentration is prepared. 3) 20 to 30 μl of the equimolar peptide mixture is delivered into a cuvette in the fluorimeter which contains 3 ml of aqueous buffer, after rapid mixing, fluorescence emission between 300 nm to 550 nm is monitored (excitation λ = 281 nm).

Induction of energy transfer by fibril formation.

Fibrils composed of Trp-Aβ(9-25) alone, AEDANS-Aβ(9-25) alone, and equimolar mixtures of the two peptides were formed by diluting concentrated solutions of the peptides dissolved in 40% TFE into aqueous buffer. The fluorescence emission spectrum of these peptide preparations are shown in Figure 1. The emission of the Trp fluorophore is significantly quenched in the mixed fibril preparation compared to the Trp-emission of fibrils composed of Trp-Aβ(9-25) alone. The AEDANS-emission, on the other hand, is significantly

enhanced in the mixed fibril preparation, relative to the emission of fibrils composed of AEDANS-Aβ(9-25) alone. Donor quenching of Trp and the sensitized emission of AEDANS are typical features of energy transfer.

pH dependence of fibril formation.

It is known that fibril formation is pH dependent. To study this effect using fluorescence energy transfer, spectra were collected at varying pH. AEDANS intensities from 425-525 nm were integrated and this value was expressed as a ratio versus the integrated intensities of Trp from 340-375 nm. The degree of fibril formation would thus be directly proportional to this acceptor/donor ratio. The advantages of this manipulation are two-fold: 1) taking the ratios diminishes the within experiment errors such as noise in the intensity measurements, and 2) results can now be compared between experiments because the acceptor/donor ratio is concentration-independent and it depends only on the fraction of Aβ(9-25) molecules in multimeric states.

Fibril formation reached a maximum at pH 5 and decreased both towards more acidic and more basic pH (Figure 2). Basal acceptor/donor ratios occurred below pH 2 in the acidic limb and above pH 8 in the basic limb of the titrations suggesting very few fibrils were present beyond these values. The pH dependence of fibril formation was modelled as a sequential equilibrium between 4 ionic species, and the data were fit to the following equation (I) which relates the acceptor/donor ratio to the concentrations of each ionic species and 3 pKa constants:

(I)

$$\frac{\text{acceptor}}{\text{donor}}\ \text{ratio} = \frac{\left[10^{(-2\,pH-pK\,\text{baseline}2)} + 10^{(-pH-pK2)\,\text{baseline}3} + 10^{(-pK1-pK2-pK3\,\text{baseline}4)} + 10^{(-3\,pH)\,\text{baseline}1}\right]}{10^{(-2\,pH-pK3)} + 10^{(-pH-pK1-pK2)} + 10^{(-pK1-pK2-pK3)} + 10^{(-3pH)}}$$

The fitted equation gave apparent pKa's of 3.98, 5.86 and 7.62.

The pH titration in the presence of 500 mM NaCl showed a dramatic decrease in the level of maximum fibrillogenesis (Figure 2). The salt titration curve at pH 5.25 demonstrated the inverse relationship between NaCl concentration and fibril formation (Figure 2 inset).

The effect of pH on fibrillogenesis was also studied using electron microscopy. The analysis indicates that equimolar mixtures of Trp/AEDANS-Aβ(9-25) give rise to fibrils that are several mm long and approximately 60 Å in diameter. The morphology of these fibrils are very similar to fibrils formed by Aβ(11-25) (Fraser et al., 1994) and by full-length Aβ (Fraser et al., 1991). The amount of fibrils in the micrographs at three different pH values 2, 5 and 8 paralleled the observations made using fluorescence, with a great number of fibrils present at pH 5 and few at pH 2 and pH 8. Furthermore, continued aging of the fibrils for 1 week showed a time-dependent change in morphology from loose short aggregates to well-structured, long fibrils with a "braided" appearance. These braided fibrils were most evident in the pH 5 samples.

Kinetics of exchange between monomers and fibrils.

Fibrils composed of Trp-Aβ(9-25) alone and AEDANS-Aβ(9-25) alone were formed separately, aged for 24 hrs, mixed, and then fluorescence emission spectra of the mixed fibril preparation were acquired at timed intervals for the next 8 hrs. The acceptor/donor ratio as a function of time after mixing is shown in Figure 3.

Fluorescent fibrillogenesis assay.

The successful development of a fluorescent fibrillogenesis assay depends on two prerequisites: a)

the extrinsic fluorescent groups must not interfere with the process of fibril formation, and b) significant energy transfer must occur between donor and acceptor fluorophores in the fibril state. The data presented show that both prerequisites have been met. The fluorescent-labelled Aβ(9-25) peptides formed fibrils, as observed by electron microscopy, that had very similar morphology to fibrils formed by native Aβ peptides and Aβ fragments (Fraser et al., 1991; Fraser et al., 1994). Significant donor quenching of Trp and sensitized emission of AEDANS were observed with fibrils composed of equimolar mixtures of Trp- and AEDANS-Aβ(9-25), compared to fibrils composed of Trp-Aβ(9-25) alone and AEDANS-Aβ(9-25) alone (Figure 1). These features are typical of energy transfer processes; therefore, supporting the conclusion that energy transfer was induced by fibril formation in the mixed fibril preparation. The dependence of the acceptor/donor ratio on pH (Figure 2) correlated with the electron microscopic observations of the number of fibrils present in solutions of Trp- and AEDANS-Aβ(9-25) at different values of pH. This correlation cross-validates the fluorescent fibrillogenesis assay with electron microscopy, and it indicates that the acceptor/donor ratio is a quantitative measure of the fraction of Aβ(9-25) molecules present in the fibril state.

pH dependence of fibrillogenesis

FTIR and electron microscopy studies have established that Aβ fibrillogenesis is very pH dependent; thus, it was concluded that fibrillogenesis is stabilized, at least in part, by electrostatic interactions (Fraser et al., 1991; Fraser et al., 1994). However, the number and type of ionizable groups involved in the electrostatic interaction has not been determined. The sensitive and quantitative nature of the fluorescent fibrillogenesis assay allows such quantitation of the pH

dependence. The simplest model (equation I) that accurately describes the pH effects shown in Figure 2 is a sequential equilibrium between 4 ionic species, where each species differ in the ionization state of three chemical groups. The fitted equation gave apparent pKa's of 3.98, 5.86 and 7.62. The apparent pKa of 3.98 is suggestive of carboxyl groups on Asp and Glu. The apparent pKa's of 5.86 and 7.62 is suggestive of imidazole groups on His. In light of this information one can conclude that fibrillogenesis in Aβ(9-25) is maximal when His13, His 14, Glu11, Glu22 and Asp23 are in their ionized states. The involvement of Lys16 cannot be assessed because fibril formation does not occur around pH 10 where titration of Lys sidechain occurs.

As regards the mechanism of electrostatic stabilization of fibrillogenesis, two different scenarios could apply: the pH dependence could be caused by either isoelectric precipitation or formation of salt bridges. In the case of isoelectric precipitation, the maximal fibrillogenesis at pH 5.00 (Figure 2) is a result of the peptide showing a greater tendency to associate at its isoelectric point. Alternatively, the pH dependence could be caused by formation of imidazole-carboxylate salt bridges between His sidechains and sidechains of Asp and/or Glu. To differentiate between these two possibilities the effect of counter ion screening by NaCl was examined. If the pH dependence of fibrillogenesis is an isoelectric effect, then addition of NaCl to the solution should screen the repulsive interactions between peptides at pH values that are away from the isoelectric point causing a broadening of the pH titration curve. If, on the other hand, the pH dependence is caused by formation of imidazole-carboxylate salt bridges, then addition of NaCl will screen these attractive interactions and the pH titration curve will be attenuated. Addition of 0.5 M NaCl resulted in

attenuation of the pH titration curve, supporting the conclusion that imidazole-carboxylate salt bridges contribute to the stability of the fibril formed by Aβ(9-25). This conclusion was further substantiated by demonstrating that NaCl titration causes a reduction in fibrillogenesis (Figure 2 inset). In addition, since two imidazole titrations were detected, it can also be concluded that there are two stabilizing imidazole-carboxylate salt bridges per monomer.

Kinetics of exchange between monomers and fibrils.

In the exchange experiments, fibrils of Trp-Aβ(9-25) and AEDANS-Aβ(9-25) were formed separately and then mixed. Upon mixing these fibrils, little if any energy transfer was expected because fibrils would be composed of either Trp-Aβ(9-25) or AEDANS-Aβ(9-25), but not both. Interaction between fluorophores would be limited by fibril structure. This indeed was the case; immediately after mixing, the acceptor/donor ratio was only slightly greater than the basal ratio (Figure 3). However, the acceptor/donor ratio increased significantly over the next 8 hrs, which suggested that fibrils, though insoluble, existed in a dynamic state in which monomers were in constant exchange between fibrils. The kinetics of exchange was adequately fit to two exponentials, which had relaxation times of 21 seconds and 77 minutes (Figure 3).

The use of amyloid-enhancing factors to promote fibril formation under physiological conditions.

Studies of the pH dependence of Aβ fibrillogenesis have revealed that fibrils do not readily form at the physiological pH of 7.00. Amyloid-enhancing factors such as heparin are known, however, to promote fibril formation under physiological conditions (Fraser et al., 1993). Thus, by using the fluorescent fibrillogenesis assay in the presence of an amyloid-enhancing factor, Aβ

fibrillogenesis can be investigated at the physiological pH of 7.00. As shown in Figure 4, increasing the amount of heparin present results in an increased level of fluorescence energy transfer as evidenced by a decrease in donor fluorescence. The data shown in Figure 4 were obtained using a concentration of Trp/AEDANS-Aβ(9-25) of 100 nM, and the buffer was phosphate-buffered saline, pH 7.00.

The invention is a new technique for monitoring fibrillogenesis by Aβ. Using this technique, it has been demonstrated that fibril formation by Trp-Aβ(9-25) and AEDANS-Aβ(9-25) is stabilized by 2 imidazole-carboxylate salt bridges. It has also been demonstrated that monomers that compose the fibril are not kinetically trapped in the fibril state, and that they exchange with the soluble fraction. The exchange kinetics are multiphasic. This information will be extremely valuable when designing pharmacological agents that inhibit fibril formation. By obtaining a greater understanding of the fibril assembly process under physiological conditions, a better idea of which features of the process are the most susceptible to attack can be obtained and exploited.

The standardized fluorescence assay for fibrillogenesis of the invention should also be of immediate value for screening compounds that have potential for being inhibitors of fibril formation.

MATERIALS AND METHODS

Peptide synthesis and fluorescent labeling

Peptides were synthesized, by the solid phase method on the Milligen 9050™ peptide synthesizer, as peptide-amides using Rink-resin (Advanced Chemtech). An active ester coupling procedure, employing pentafluorophenyl esters of 9-fluorenylmethoxycarbonyl amino acids, was used. The N-termini was acetylated with acetic anhydride.

The peptides were cleaved from the resin with 95:2.5:2.5 trifluoroacetic acid: thioanisole: ethanedithiol mixture. The peptides were purified by C_{18} reverse phase chromatography, and peptide identity was confirmed by FAB mass spectrometry and amino acid analysis. Peptide purity was assessed by analytical C_{18} reverse phase chromatography using the Pharmacia FPLC system.

5-Acetylethyldiaminonaphthalene-1-sulfonic acid (AEDANS) labelling was performed by incubation of 1 mM Cys-containing peptide with 5 mM (1,5)-IAEDANS (Molecular Probes) in 50 mM Tris-HCl, 6 M guanidine hydrochloride, pH 8.0 for 18 hrs. AEDANS-labelled peptide was separated from unreacted peptide and 1,5-IAEDANS by C_{18} reverse phase chromatography.

Preparation of peptide stock solutions

The lyophilised Trp-Aβ(9-25) was dissolved in a 2 ml solution of 40% (v/v) trifluoroethanol (TFE) containing 10 mM acetic acid. This stock solution was diluted 1:10 with 7.5 M guanidine hydrochloride (GdnHCl) and the peptide concentration was determined via tryptophan absorbance at 280 nm with an absorbance coefficient (ϵ) of 5690 $M^{-1}cm^{-1}$ (Edelhoch, 1967).

Lyophilised AEDANS-Aβ(9-25) was dissolved in a 2ml solution of 40% (v/v) TFE adjusted to pH 9 with ammonium hydroxide. Hudson and Weber have shown that ϵ for AEDANS in 40% ethanol is 6500 $M^{-1}cm^{-1}$ and the absorbance λ_{max} is 338.0 nm. It was found that the difference in absorbance between AEDANS in ethanol and TFE is less than 1%, well within experimental error; therefore, an ϵ of 6500 $M^{-1}cm^{-1}$ were used in all concentration determinations of AEDANS-Aβ(9-25) in 40% TFE.

Absorbance measurements were made on a Perkin Elmer Lambda 3B™ spectrophotometer. The stock solutions were

stored at -20°C.

Electron Microscopy

Negatively stained fibrils were prepared by floating charged pioloform, carbon-coated grids on peptide solutions (0.025 mg/mL Trp:Aβ(9-25) and 0.025 mg/mL AEDANS:Aβ(9-25), pH2-pH8). These solutions were pre-aged 24 h and 1 week. To control pH, the peptide solutions were made using a buffer of 1 mM borate, 1 mM citrate and 1 mM phosphate. After the grids were blotted and air-dried, the samples were stained with 1% (w/v) phosphotungstic acid which was prepared using the same borate, citrate, phosphate buffer and pH adjusted to correspond to the respective samples. The Hitachi H-7000™ instrument used for visualization was operated at 75 kV. The samples were inspected and images of representative fibrils were recorded.

Fluorescence Spectroscopy

Steady-state fluorescence was measured at room temperature using a Photon Technologies International QM-1™ fluorescence spectrophotometer equipped with excitation intensity correction, a temperature controlled cell holder and a magnetic stirrer. Emission spectra from 300nm to 550 nm were collected with excitation set at 281 nm. Both the excitation and emission monochromator slit widths were 4 nm.

In the energy transfer experiments, four spectra were taken: a blank of the buffer containing 1 mM borate, 1 mM citrate and 1 mM phosphate; 3 μM Trp:Aβ(9-25) alone; 3 μM AEDANS:Aβ(9-25) alone; and a mixture of 3μM of each labeled peptide. The samples were pre-aged at pH 5 for 18 to 24 hours prior to spectral acquisition. The pH titrations were performed using hydrochloric acid and sodium hydroxide. For the salt studies, samples were pre-aged with the appropriate

concentration of sodium chloride.

In the exchange experiments 6 μM Trp:Aβ(9-25) and 6 μM AEDANS:Aβ(9-25) were incubated separately overnight at pH 5. These solutions were mixed giving a final concentration of 3 μM each labeled peptide at which time spectra were collected.

REFERENCES

Barrow, C.J., and Zagorski, M.G. (1991) Solution structures of β-peptide and its constituent fragments: relation to amyloid deposition. Science 253, 179-182.

Crowther, R.A. (1991) Structural aspects of pathology in Alzheimer's disease. Biochim. Biophys. Acta 1096, 1-9.

Edelhoch, H. (1967) Biochemistry 6, 1948 - 1954.

Fairclough, R.H., and Cantor, C.R. (1978) The use of singlet-singlet energy transfer to study macromolecular assemblies. Methods Enzymol. 48, 347-379.

Fraser, P.E., Nguyen, J.T., Surewicz, W.K., and Kirschner, D.A. (1991) pH-dependent structural transitions of Alzheimer amyloid β/A4 peptides. Biophysical J. 60, 1190-1201.

Fraser, P.E., Nguyen, J.T., Inouye, H., Surewicz, W.K., Selkoe, D.J., Podlisny, M.B., and Kirschner, D.A. (1992) Fibril formation by primate, rodent, and Dutch-hemorrahagic analogues of Alzheimer amyloid β-protein. Biochemistry 31, 10716-10723.

Fraser, P.E., Levesque, L., and McLachlan, D.R. (1993) Biochemistry of Alzheimer's Disease Amyloid Plaques. Clin. Biochem. 26, 339-349.

Fraser, P.E., McLachlan, D.R., Surewicz, W.K., Mizzen, C.A., Snow, A.D., Nguyen, J.T., and Kirschner, D.A. (1994) Conformation and fibrillogenesis of Alzheimer Aβ peptides with selected substitutions of charged residues. J. Mol. Biol. (in the press).

Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., Blackwell, C., Carr, T., Clemens, J., Donaldson, T., Gillespie, F., et al. (1995) Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. Nature 373, 523-527.

Goate A. Chartier-Harlin MC. Mullan M. Brown J. Crawford F. Fidani L. Giuffra L. Haynes A. Irving N. James L. et al. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349, 704-706.

Haass, C., Schlossmacher, M.G., Hung, A.Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B.L., Lieberburg, I., Koo, E.H., Schenk, D., Teplow, D.B., and Selkoe, D.J. (1992) Amyloid β-protein is produced by cultured cells during normal metabolism. Nature 359, 322-325.

Halverson, K., Fraser, P.E., Kirschner, D.A., and Lansbury, P.T. (1990) Molecular determinants of amyloid deposition in Alzheimer's disease: conformational studies of synthetic beta-protein fragments. Biochemistry 29, 2639-2644.

Hilbich, C., Kister-Wolke, B., Reed, J., Masters, C.L., and Beyreuther, K. (1992) Substitutions of hydrophobic amino acids reduce the amyloidogenicity of Alzheimer's disease βA4 peptides. J. Mol. Biol. 228, 460-473.

Mattson, M.P., Barger, S.W., Cheng, B., Lieberburg, I., Smith-Swintosky, V.L., and Rydel, R.E. (1993) β-Amyloid precursor protein metabolites and loss of neuronal Ca^{2+} homeostasis in Alzheimer's disease. Trends Neurochem. Sci. 16, 409-414.

Pike C.J., Burdick, D., Walencewicz, A.J., Glabe, C.G., Cotman, C.W., (1993) Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. J. Neurosci. 13, 1676-1687.

Selkoe, D.J. (1993) Physiological production of the β-amyloid protein and the mechanism of Alzheimer's disease. Trends Neurochem. Sci. 16, 403-409.

Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Sinha, S., Schlossmacher, M.G., Whaley, J., Swindlehurst, C., McCormack, R., Wolfert, R., Selkoe, D.J., Lieberburg, I., and Schenk, D. (1992) Isolation and quantification of soluble Alzheimer's β-peptide from biological fluids. Nature 359, 325-327.

Yankner, B.A., Dawes, L.R., Fisher, S., et al. (1989) Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's disease. Science 245, 417-420.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: THE ONTARIO CANCER INSTITUTE

(ii) TITLE OF INVENTION: PROTEIN FIBRIL ASSEMBLY ASSAY

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Ridout & Maybee
(B) STREET: Suite 2400, One Queen Street East
(C) CITY: Toronto
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) ZIP CODE: M5C 3B1

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette - 3.5 inch, 1.4 Mb storage
(B) COMPUTER: IBM PC Compatible
(C) OPERATING SYSTEM: MS-DOS 6.00
(D) SOFTWARE: Wordperfect 5.1

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Lake, James R.
(B) REFERENCE/DOCKET NUMBER: ONTCA/46B

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (416) 868-1482
(B) TELEFAX: (416) 362-0823

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acid residues
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Tyr Glu Val His His Glu Lys Leu Val Phe Phe Ala Glu Asp Val Gly
5 10 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ix) FEATURE:
(D) OTHER INFORMATION: The N-terminal amino acid residue Xaa is AcTRP, and the C-terminal amino acid residue Xaa is Gly-$CONH_2$.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa Gly Gly Tyr Glu Val His His Gln Lys Leu Val Phe Phe
5 10
Ala Glu Asp Val Xaa
15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ix) FEATURE:
(D) OTHER INFORMATION: The N-terminal amino acid residue Xaa is AcCYS (AEDANS), and the C-terminal amino acid residue Xaa is Gly-$CONH_2$.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Xaa Gly Gly Tyr Glu Val His His Gln Lys Leu Val Phe Phe
5 10
Ala Glu Asp Val Xaa
15

CLAIMS:

1. A method for *in vitro* monitoring of peptide or protein fibril assembly, comprising the steps of:

1) attaching a donor fluorophore to a first fibrillogenic protein or peptide;

2) attaching an acceptor fluorophore to a second fibrillogenic protein or peptide, the donor and acceptor fluorophores being located on said first and second peptides or proteins so that they are juxtaposed to permit energy transfer between them upon fibril formation;

3) mixing the first and second fluorophore-containing peptides or proteins in solution at an approximately normal physiological concentration; and

4) monitoring formation of fibrils by observing the occurrence of fluorescence energy transfer between the donor and acceptor fluorophores upon excitation of the donor fluorophores.

2. A method as claimed in claim 1, wherein the donor fluorophore is a tryptophan residue.

3. A method as claimed in claim 2, wherein the donor fluorophore is attached to the N-terminus of a fibrillogenic peptide or protein.

4. A method as claimed in claim 3, wherein the peptide or protein is modified by adding a glycine residue to the N-terminus prior to attaching the fluorophore.

5. A method as claimed in claim 1, wherein the acceptor fluorophore is an AEDANS residue.

6. A method as claimed in claim 5, wherein the acceptor fluorophore is attached to a cysteine sulfhydryl at the N-terminus of a fibrillogenic peptide or protein.

7. A method as claimed in claim 6, wherein the peptide or protein is further modified by providing a glycine residue following the N-terminal cysteine.

8. A method as claimed in claim 1, wherein the mixing step comprises:

a) preparing concentrated stock solutions of said first and second fluorophore-containing peptides or proteins in denaturant and accurately determining the concentrations of the peptides or proteins by ultraviolet (UV) absorbance measurements of the solutions using the known absorption coefficients of the fluorophores; and

b) mixing appropriate volumes of each solution to produce equimolar concentrations of each peptide or protein in denaturant solution.

9. A method as claimed in claim 8, wherein the monitoring step includes diluting out the denaturant, thereby initiating fibril assembly.

10. A method as claimed in claim 1, wherein the mixing step includes the addition of a promoter at a concentration sufficient to promote fibril formation.

11. A method as claimed in claim 10, wherein the promoter is heparin.

12. A method as claimed in claim 11, wherein the heparin concentration is 0.1-1 mg/ml.

13. A method as claimed in claim 12, wherein the method

is carried out at physiological pH.

14. A method as claimed in claim 1, wherein the monitoring step is carried out in a fluorimeter.

15. A method as claimed in claim 8, wherein the denaturant is approximately 40% trifluoroethanol, and each peptide or fragment is dissolved in the denaturant at a millimolar concentration.

16. A method as claimed in claim 15, wherein the monitoring step includes diluting out the denaturant by quickly adding 20-30 µl of the equimolar mixture to about 3 ml of aqueous buffer.

17. A method as claimed in claim 1, comprising the further step of mixing the first and second fluorophore-containing peptides or proteins in the presence of a candidate inhibitor of fibril formation.

18. A method for *in vitro* monitoring of Alzheimer β-amyloid protein fibril assembly, comprising the steps of:

1) attaching a donor fluorophore to a first Alzheimer β-amyloid peptide or to a fibrillogenic fragment of Alzheimer β-amyloid peptide;

2) attaching an acceptor fluorophore to a second Alzheimer β-amyloid peptide or to a fibrillogenic fragment of Alzheimer β-amyloid peptide, the donor and acceptor fluorophores being located on said first and second peptides or fragments so that they are juxtaposed to permit energy transfer between them upon fibril formation;

3) mixing the first and second fluorophore-

containing peptides or fragments in solution at an approximately normal physiological concentration; and

4) monitoring formation of fibrils by observing the occurrence of fluorescence energy transfer between the donor and acceptor fluorophores upon excitation of the donor fluorophores.

19. A method as claimed in claim 18, wherein the donor fluorophore is a tryptophan residue.

20. A method as claimed in claim 19, wherein the donor fluorophore is attached to the N-terminus of Alzheimer β-amyloid peptide or a fibrillogenic fragment of Alzheimer β-amyloid peptide.

21. A method as claimed in claim 20, wherein Trp-Aβ(9-25), SEQ ID NO:2, is said first fluorophore-containing fragment of Alzheimer β-amyloid peptide.

22. A method as claimed in claim 21, wherein the peptide or peptide fragment is modified by adding a glycine residue to the N-terminus prior to attaching the fluorophore.

23. A method as claimed in claim 18, wherein the acceptor fluorophore is an AEDANS residue.

24. A method as claimed in claim 23, wherein the acceptor fluorophore is attached to a cysteine sulfhydryl at the N-terminus of Alzheimer β-amyloid peptide or a fibrillogenic fragment of Alzheimer β-amyloid peptide.

25. A method as claimed in claim 24, wherein the peptide or peptide fragment is further modified by providing a glycine residue between the N-terminal cysteine and Gly 9 residues.

26. A method as claimed in claim 25, wherein AEDANS-Aβ(9-25), SEQ ID NO:3, is said second fluorophore-containing fragment of Alzheimer β-amyloid peptide.

27. A method as claimed in claim 18, wherein the mixing step comprises:

a) preparing concentrated stock solutions of said first and second fluorophore-containing peptides or fragments in denaturant and accurately determining the concentrations of the peptides or fragments by ultraviolet (UV) absorbance measurements of the solutions using the known absorption coefficients of the fluorophores; and

b) mixing appropriate volumes of each solution to produce equimolar concentrations of each peptide or fragment in denaturant solution.

28. A method as claimed in claim 27, wherein the monitoring step includes diluting out the denaturant, thereby initiating fibril assembly.

29. A method as claimed in claim 18, wherein the mixing step includes the addition of a promoter at a concentration sufficient to promote fibril formation.

30. A method as claimed in claim 29, wherein the promoter is heparin.

31. A method as claimed in claim 30, wherein the heparin concentration is 0.1-1 mg/ml.

32. A method as claimed in claim 31, wherein the method is carried out at physiological pH.

33. A method as claimed in claim 18, wherein the

monitoring step is carried out in a fluorimeter.

34. A method as claimed in claim 27, wherein the denaturant is approximately 40% trifluoroethanol, and each peptide or fragment is dissolved in the denaturant at a millimolar concentration.

35. A method as claimed in claim 34, wherein the monitoring step includes diluting out the denaturant by quickly adding 20-30 μl of the equimolar mixture to about 3 ml of aqueous buffer.

36. A method as claimed in claim 35, wherein the fluorophore-containing peptides are Trp-Aβ(9-25), SEQ ID NO:2, and AEDANS-Aβ(9-25), SEQ ID NO:3, and fibril formation is monitored by measuring emission in the range 300-550 nm using a fluorimeter with excitation at 281 nm.

37. A method as claimed in claim 18, comprising the further step of mixing the first and second fluorophore-containing peptides or fragments in the presence of a candidate inhibitor of amyloid fibril formation.

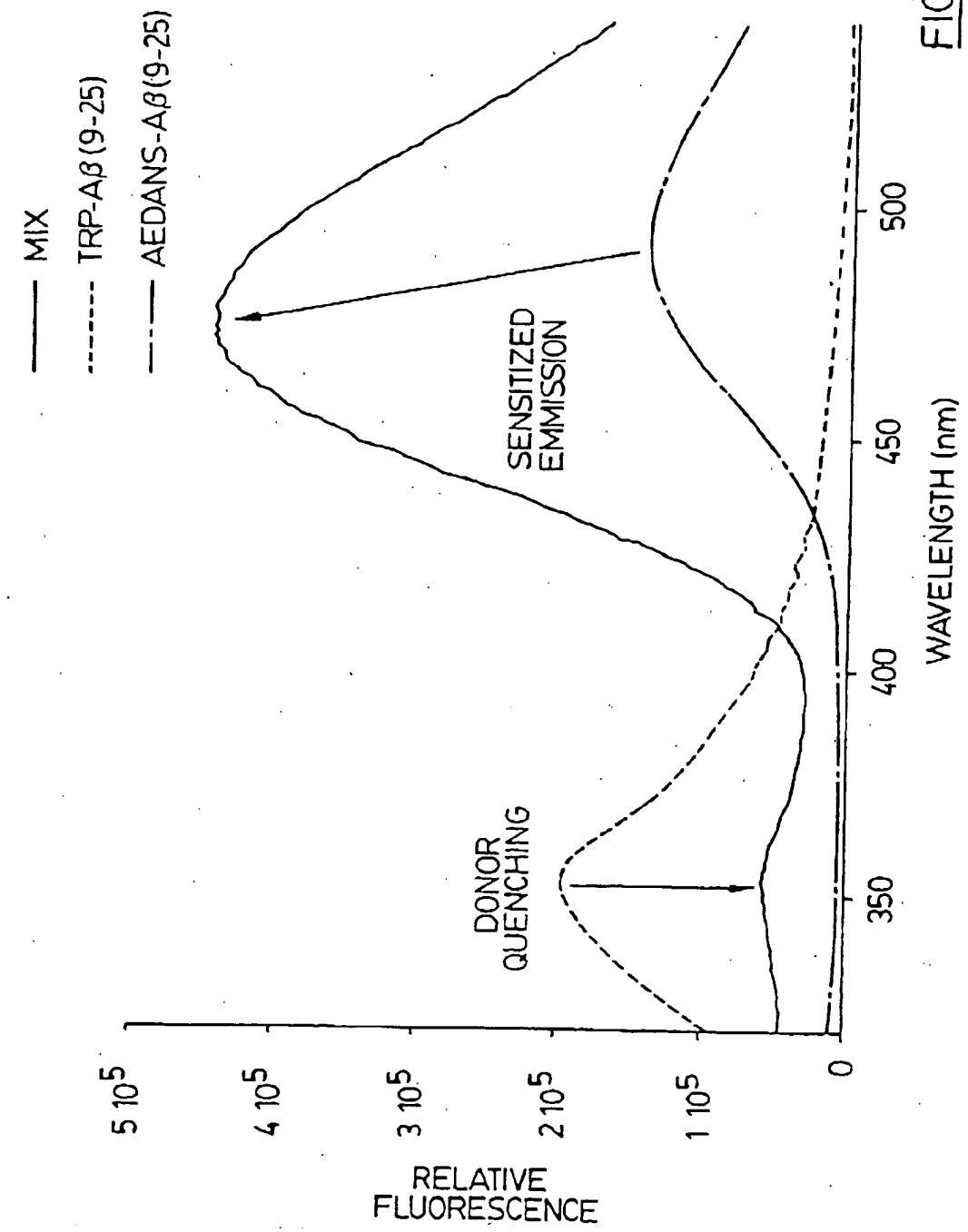

MIX
TRP-Aβ(9-25)
AEDANS-Aβ(9-25)
SENSITIZED EMMISSION
DONOR QUENCHING
5 10^5
4 10^5
3 10^5
2 10^5
1 10^5
0
RELATIVE FLUORESCENCE
350
400
450
500
WAVELENGTH (nm)

FIG. 1

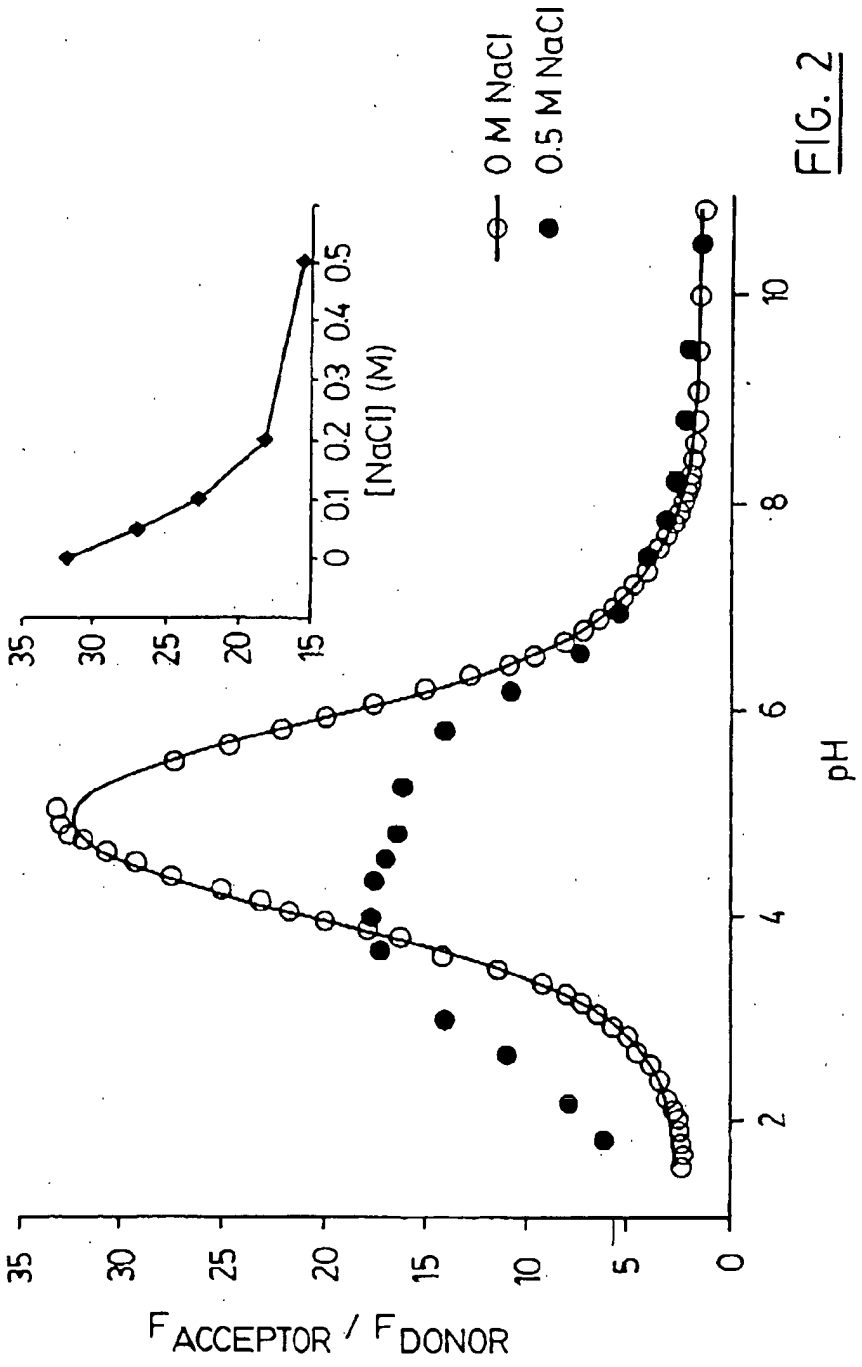
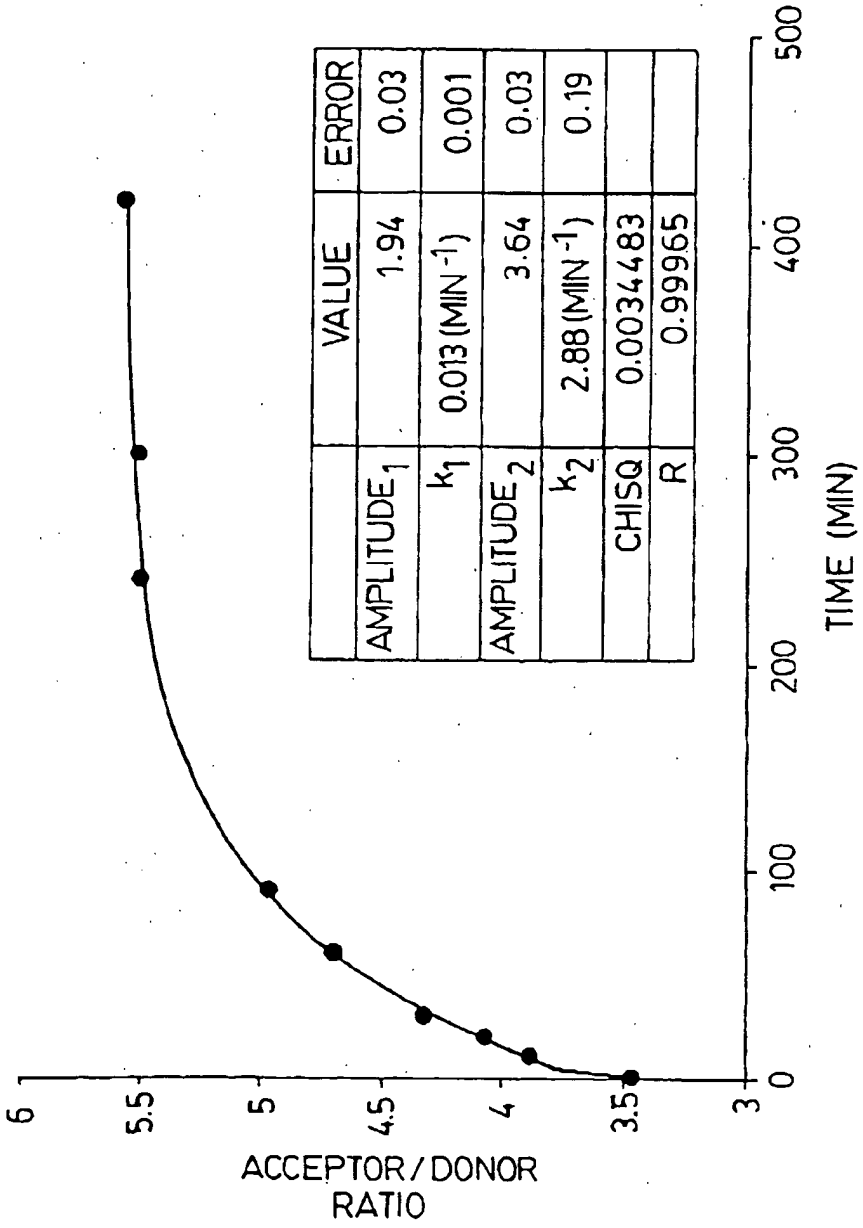

0 M NaCl
0.5 M NaCl
[NaCl] (M)
0 0.1 0.2 0.3 0.4 0.5
35 30 25 20 15
pH
2 4 6 8 10
F ACCEPTOR / F DONOR
35 30 25 20 15 10 5 0

FIG. 2

FIG. 3


VALUE
ERROR
AMPLITUDE1
1.94
0.03
k1
0.013 (MIN-1)
0.001
AMPLITUDE2
3.64
0.03
k2
2.88 (MIN-1)
0.19
CHISQ
0.0034483
R
0.99965
6
5.5
5
4.5
4
3.5
3
ACCEPTOR / DONOR RATIO
0
100
200
300
400
500
TIME (MIN)

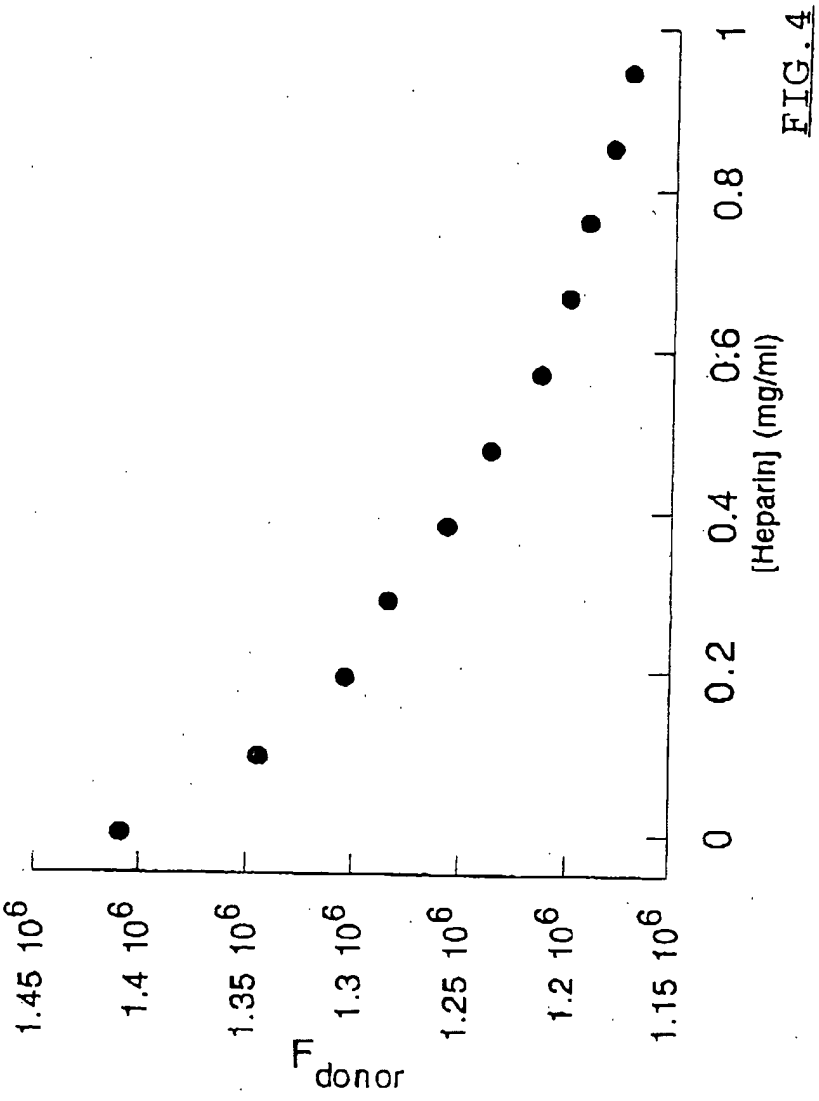

1.45 10^6
1.4 10^6
1.35 10^6
1.3 10^6
1.25 10^6
1.2 10^6
1.15 10^6
F donor
0
0.2
0.4
0.6
0.8
1
[Heparin] (mg/ml)

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No
PL./CA 96/00555

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 04194 (UNIV MINNESOTA ;HARVARD COLLEGE (US)) 4 March 1993 ---	
A	EP,A,0 552 108 (UNIV MARYLAND) 21 July 1993 ---	
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 1, 9 January 1992, pages 546-554, XP002021280 D. BURDICK ET AL.: "Assembly and aggregation properties of synthetic alzheimer's a4/beta amyloid peptide analogs." --- -/--	

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 December 1996	10. 01. 97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Cartagena y Abella,P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 96/00555

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROTEIN SCIENCE, vol. 2, March 1993, pages 404-410, XP000613188 H. LEVINE : "Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: detection of amyloid aggregation in solution." ---	
P,A	BIOCHEMISTRY, vol. 35, 21 May 1996, pages 6470-6482, XP002021281 Z. LAI ET AL.: "The acid-mediated denaturation pathway of transthyretin yields a conformational intermediate that can self-assemble into amyloid." ---	
P,A	PROC. NATL. ACAD. SCI. USA, vol. 93, 9 January 1996, pages 452-455, XP002021282 B. SOLOMON ET AL.: "Monoclonal antibodies inhibit in vitro fibrillar aggregation of the Alzheimer beta-amyloid peptide." -----	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

nformation on patent family members

International Application No
PL./CA 96/00555

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9304194	04-03-93	US-A-	5434050	18-07-95
		EP-A-	0599979	08-06-94
		JP-T-	6510761	01-12-94
EP-A-0552108	21-07-93	CA-A-	2087413	18-07-93
		JP-A-	6066802	11-03-94

Form PCT/ISA/210 (patent family annex) (July 1992)